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PRINCIPAL INVESTIGATOR: Carolyn R. Chapman

CONTRACTING ORGANIZATION: Harvard Medical School
Boston, Massachusetts 02115

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FOREWORD

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Carolyn R. Chapman 4/14/90
PI - Signature Date

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Introduction

I. Ataxia-Telangiectasia: Elevated Cancer Risk

Ataxia-telangiectasia (A-T) is a debilitating and progressive autosomal recessive human disease in which homozygotes suffer a predisposition to cancer, such as lymphomas and leukemias, and a marked sensitivity to ionizing radiation. In addition, the patients exhibit a variety of other symptoms; in particular, the immune system, nervous system, and skin are affected (1)(2).

Certain studies have found that individuals heterozygous for ataxia-telangiectasia have an increased risk of cancer (3); in particular, female heterozygotes may be five times more likely to develop breast cancer than control populations (4). However, this point is still not resolved, as some groups have found that ATM mutations do not confer a predisposition to early onset of breast cancer (5). It is clear that research on the biology of the A-T gene is likely to increase our understanding of one potential cause of breast cancer and may improve our ability to provide the most appropriate methods of diagnosis and treatment for these patients.

II. The *ATM* Gene: Homologous to *S. pombe rad3⁺*

ATM (ataxia-telangiectasia mutated), the gene responsible for causing ataxia-telangiectasia, has been cloned and sequenced (6, 7). ATMp is homologous to a family of proteins which includes *S. pombe* Rad3p (the gene product of the *S. pombe rad3⁺* gene) (7, 8). Both ATMp (the predicted protein product of the *ATM* gene) and Rad3p contain leucine zipper motifs towards their N-termini, which may mediate their dimerization or interaction with other proteins (6). They also each contain a kinase domain at their C-termini (7). Rad3p has been shown to be capable of autophosphorylation (8), while ATM has been shown to be capable of phosphorylating protein substrates (9). *ATM* and *rad3⁺* are also homologous to the *S. cerevisiae MEC1/ESR1* gene (7). In addition to sharing substantial sequence homology, *ATM*-deficient cells and *rad3⁻* and *mec1⁻* mutants exhibit remarkable phenotypic similarities.

III. The *ATM* gene family: Functionally and structurally similar proteins in a range of organisms

Human *ATM*, *S. pombe rad3⁺* and *S. cerevisiae MEC1* are cell cycle checkpoint genes. These genes maintain the proper order of events in the cell cycle by preventing cell cycle progression at inappropriate times (10). For example, to ensure that a euploid set of chromosomes is transferred to the next generation, DNA synthesis must be completed before cells divide. To maintain the fidelity of genetic information, cells must not initiate mitosis in the presence of damaged DNA. Both *rad3⁻* and *mec1⁻* mutants fail to maintain these dependencies (11)(12)(13)(14). Although the checkpoint deficiency is most well-studied at G2/M in these organisms, there is evidence that the cells lack other cell cycle controls as well. Compared to wild-type cells, *mec1⁻* mutants exhibit a reduced ability to slow DNA replication in response to alkylating agents (15). This finding suggests that the *MEC1* checkpoint normally functions not only to block mitosis, but also to slow DNA synthesis in the presence of DNA damage.

A-T cells also lack the ability to execute a number of checkpoint controls which normally act to prevent cell cycle progression in the presence of damaged DNA (2)(16). These cells lack a G1/S checkpoint, the ability to delay entry into S phase in the presence of DNA damage induced by ionizing radiation. A-T cells also exhibit a defect in their G2/M checkpoint, which prevents cells with damaged DNA from entering mitosis (17)(18). Also like *mec1⁻* mutants, A-T cells exhibit a deficiency in slowing the rate of DNA synthesis following exposure to DNA-damaging agents such as ionizing radiation (19)(20).

The above evidence suggests that the *ATM*, *rad3⁺*, and *MEC1* gene products work by similar mechanisms to detect and respond to DNA damage by activating repair pathways and arresting cell cycle progression.

The ATMp family appears to define a novel class of proteins involved in communicating information about DNA to the cell. Their function is undoubtedly extremely important, as evidenced by the drastic phenotypes caused by loss-of-function mutations in these genes. I have initiated genetic and biochemical analysis to elucidate the molecular mechanism of action of the *rad3⁺* gene. Any information we gain about *rad3⁺* can be applied to further our understanding of the functions of *ATM*.

IV. The *Schizosaccharomyces pombe* checkpoint pathway

rad3+ is one of a number of checkpoint genes that have been identified in *Schizosaccharomyces pombe* (for a recent review, see (21)). The *S. pombe* checkpoint *rad* mutants, which include *rad1*, *rad3*, *rad9*, *rad17*, *rad26*, and *hus1*, all have similar phenotypes; they fail to arrest the cell cycle in the presence of either DNA damage or incomplete DNA replication. *chk1* and *cds1* encode kinases which also function in checkpoint control in fission yeast (22)(23). In contrast to the *checkpoint rad* mutants, *chk1* mutants appear to be specifically defective in responding to DNA damage, while *cds1* mutants exhibit the reciprocal problem, failure to recover from S phase arrest. Chk1p and Cds1p function downstream of the *checkpoint rad* genes (22)(23).

Rad3p is believed to play a central role in cell cycle checkpoint control in *Schizosaccharomyces pombe*. Evidence suggests that Rad3p is integrally involved in monitoring the DNA and relaying information about DNA damage and incomplete DNA replication to other cellular proteins such as DNA repair enzymes and the cell cycle machinery. My studies are aimed at achieving a better understanding of how *S. pombe rad3+* functions, as a model system for understanding the biology of human *ATM*.

Body

I. Identification of gene products that interact with Rad3p

i. Screen for High Copy Suppressors of *rad3⁻* mutants

One of my aims is to identify other proteins which interact with Rad3p or which are in the Rad3p signal transduction pathway. My first strategy to do this was to screen for high copy suppressors of *rad3⁻* mutants by looking for complementation of their hydroxyurea (HU) sensitive phenotype. Of 36,000 colonies screened, I pursued 11 for further study. After performing sequencing analysis at the ends of some of the clones, we discovered that at least two sets of suppressors were isolated twice. We are encouraged by this fact, because it indicates that I was screening with consistency. Two of my suppressors appear to suppress my *rad3* DN strains (see below). However, in general, the isolated suppressors do not exhibit very strong suppressor phenotypes, and sequence analysis has not yet provided clues as to how the gene products could be tied into the Rad3p pathway. In the future, as the *S.pombe* genome is sequenced, more information about these genes may become available. For the time being, I have put this project on hold because we believe other aspects of my work may lead us more directly to Rad3p interactors (see below).

ii. Classical genetics: interactions between Rad3p and DNA replication proteins

• Discovery of Synthetic Dosage Phenotype between Rad3p and *pold*

We became specifically interested in investigating the relationship that Rad3p exhibited with DNA replication proteins, since Rad3p is integrally involved in monitoring the state of the DNA. In addition, we knew that Rad3p homologues, such as ATMp and Mec1p, have roles in slowing DNA replication in response to DNA damaging agents.

In an effort to identify DNA replication proteins which may interact with Rad3p, I employed a strategy called "synthetic dosage lethality," which was described by Kroll et al. (24). The rationale of this approach is similar to that of synthetic lethality, which occurs when two mutations are viable as single mutants, but the double mutant is inviable. Synthetic lethality suggests that the two proteins may interact. In synthetic dosage lethality, instead of combining two mutations which may affect the same pathway, you overexpress one gene in a strain harboring a mutation in another gene, which may have similar or

related functions. I investigated the phenotype of overexpression of *rad3*⁺ in a collection of DNA replication mutants. My rationale was that since Rad3p acts to monitor the state of the DNA, it may interact with components of the replication machinery. Overexpression of *rad3*⁺ in wild-type yeast causes the cells to appear smaller in size and to grow more slowly than vector controls. Overexpression of *rad3*⁺ in the DNA replication mutants *cdc1*, *cdc27*, *cdc20*, *cdc18* and *cdc19* does not appear to cause any obvious phenotypes compared with wild-type cells. However, overexpression of *rad3*⁺ in two out of five *cdc6* (*polδ*) temperature sensitive alleles, *cdc6-23* and *polδts1*, causes the cells to elongate at the permissive temperature, indicating that there is a synthetic dosage interaction between the two genes. The allele specificity of the synthetic dosage phenotype is intriguing, and suggests that there may be a specific interaction between Rad3p and *polδ*. The synthetic dosage phenotype appears to be specific to *rad3*⁺. Other checkpoint genes, including *rad1*, *rad9*, *rad17*, *hus1* and *cds1*, do not cause specific elongation in *cdc6* mutants, although some of the data is difficult to interpret because certain genes cause wild-type cells to lengthen as well. Therefore, we are very pleased that we have discovered an allele-specific synthetic dosage phenotype between Rad3p and *polδ*, the leading strand DNA polymerase.

•Mutations in *polδ*: Bringing PCNA into the Picture

Francesconi et al. had already sequenced three out of five of the *cdc6* alleles I have tested (25), one of which exhibits the phenotype, *polδts1*, and two which do not. *polδts1* contains two point mutations (A143V and P144S), which result in changes in two consecutive amino acids that are conserved in yeast, humans, and cow (25). This region in human *polδ* has been implicated in binding PCNA, the processivity factor for *polδ* (26, 27). The other two sequenced alleles do not exhibit the synthetic dosage phenotype and do not contain mutations in this region (instead E271K and R1064Q). To determine whether *cdc6-23* also contains a mutation in or near the PCNA binding site, I have sequenced a 400 bp region at the N-terminus of *cdc6* by PCR from genomic DNA. *cdc6-23* contained a mutation within this region (E227K), while wild-type cells and *cdc6-121* cells did not. The mutation site for *cdc6-23* mutants has more recently been confirmed by others (28). These results do not dispute nor confirm the hypothesis that an altered *polδ*/PCNA interaction may be causing the synthetic dosage phenotype with *rad3*⁺, but they encouraged to become more interested in learning more about possible interactions between Rad3p and PCNA.

- Genetic interaction between Rad3p and PCNA, the processivity factor for pol δ

I have obtained genetic evidence to support an interaction between Rad3p and PCNA. When PCNA is overexpressed, the cells lose viability over time, become very elongated, and finally their DNA becomes stringy the cells "cut." However, overexpression of Rad3p seems to change the phenotype of these cells: cells which overexpress both PCNA and Rad3p appear healthier and show higher survival rates over time. It is also very interesting to note that checkpoint mutants suppress the phenotype of OP PCNA: they no longer elongate (29) (my own unpublished data).

iii. Studying Rad3p complexes biochemically

- Development of Rad3p Antibody

In order to investigate physical interactions between the proteins we have found to genetically interact with Rad3p, we have made polyclonal antibodies to Rad3p, as well as creating epitope-tagged versions of Rad3p. In order to generate antibodies to Rad3p, I created a construct in which the C-terminal half of Rad3p was tagged with histidine residues. This construct was overexpressed in bacteria and purified in the presence of 6M Guanidine HCl using Nickel beads. We sent the protein to Cocalico Biologicals, Inc. for injection into rabbits. The polyclonal serum recognizes bacterially-produced Rad3p as well as Rad3p from extracts from yeast which are overexpressing *rad3*⁺. I have also been able to successfully immunoprecipitate Rad3p from these extracts. We are very excited that our antibodies appear to be highly specific and work well for both Western blots and immunoprecipitations, and have used our antibodies to investigate physical interactions between Rad3p and PCNA and other proteins (as described below).

- Discovery of physical interaction between Rad3p and PCNA

In order to further our studies on the interactions between Rad3p and DNA replication proteins, we wanted to investigate the possibility that Rad3p interacts directly with PCNA or pol δ . We are very excited that I have been able to consistently coimmunoprecipitate Rad3p with PCNA antibodies when both proteins are overexpressed. Unfortunately, I cannot detect the interaction when only Rad3p is overexpressed, even though endogenous levels of PCNA are relatively high. In addition, I am unable to immunoprecipitate with Rad3p antibodies and blot for PCNA, because I bring down PCNA nonspecifically in my

rad3Δ strains. I have so far been unable to detect a Rad3p and PCNA interaction using two hybrid analysis. In terms of interactions between Rad3p and pol δ , I have been unable to detect any interactions between Rad3p and pol δ using antibodies I have generated to Rad3p and tagged versions of *cdc6*, although I have not pursued this interaction vigorously since my first few experiments yielded negative results. Two hybrid analysis has also proved negative.

- Putative PCNA-binding motif in Rad3p

After communicating our preliminary results about a possible Rad3p-PCNA interaction, Stuart MacNeill pointed out a motif in Rad3p which bears significant similarity to a domain which has been identified as mediating an interaction with PCNA in a number of proteins from diverse organisms (see Appendix 1)(30). I have deleted the 9 amino acid from Rad3p and investigated the ability of this mutant (Pdel) to complement the *rad3Δ* strain, bind PCNA, and cause elongation in *cdc6* mutants. Interestingly, Pdel does not complement the hydroxyurea sensitive phenotype of the *rad3Δ* strain. However, contrary to our expectations, Pdel seems to retain its ability to bind to PCNA when overexpressed, and still causes elongation in *cdc6* mutants. When overexpressed in wild-type strains, Pdel causes the cells to become shorter, but does not cause them to become sensitive to HU. In addition, Sarah Evans in the lab has investigated the kinase activity of Pdel; the mutant does not appear to have kinase activity as determined by her assay. These results suggest that the *cdc6* synthetic dosage phenotype may not be related to the ability of Rad3p to bind to PCNA, but further experiments are designed to address this question. For example, we are interested in determining whether PCNA can suppress the synthetic dosage phenotype between pol δ and Rad3p.

II. Regulation of Rad3p protein and activity

In order to analyze the regulation of Rad3p, we are interested in determining the levels of transcript and protein, the subcellular localization, and phosphorylation of Rad3p in conditions with and without DNA damaging agents and DNA replication blocks, such as hydroxyurea. One technical barrier to these studies has been our inability to visualize endogenous Rad3p using the antibodies we have generated (see above), either in the presence or absence of DNA damaging agents or DNA replication blocks. In order to get around this, other members in my laboratory are currently using other pieces of Rad3p to generate antibodies, in the hopes that other antibodies may be able to detect endogenous

Rad3p on Western blots. We will also construct a yeast strain in which the endogenous Rad3p is epitope-tagged. We have investigated whether overexpressed Rad3p changes its mobility (perhaps due to phosphorylation) in response to DNA damaging agents, or DNA replication blocks, but have not seen any detectable changes. This is in agreement with the finding that ATM protein levels do not change over the cell cycle, or in response to DNA damaging agents (31). This finding also makes these studies on Rad3p less imperative.

As an alternative approach to understanding the regulation of Rad3p protein and activity, we have thus decided to undertake a structure/function analysis of the Rad3p protein using several assays to determine the functions different *rad3* mutants.

- Creation of dominant negative alleles of *rad3*

Tony Carr's lab has found that overexpression of alleles of *rad3* which contain point mutations in conserved residues of the kinase domain are dominant negative, that is they confer checkpoint phenotypes on wild-type cells (8). Based on this data, we wondered whether these kinase dead dominant negative alleles of *rad3* function by titrating important Rad3p interactors into nonfunctional complexes. Since the N-terminus of Rad3p contains a leucine zipper motif which may mediate protein-protein interactions, we hypothesized that overexpression of the N-terminus without the kinase domain may also exhibit dominant negative properties. To test this, I created a *rad3* deletion mutant containing the first 775 amino acids of Rad3p and expressed it in wild-type cells. The cells became sensitive to hydroxyurea, a DNA replication inhibitor, indicating that the overexpression of the N-terminus without the kinase domain is indeed dominant negative. As expected, this "Nterm" construct fails to complement *rad3*⁻ mutants. To exhibit the dominant negative phenotype, the "Nterm" construct must be extremely overexpressed; moderate overexpression does not confer dominant negative properties on wild-type cells. Overexpression of the kinase domain of Rad3p might also be a dominant negative if the N-terminus contained an important inhibitory regulatory domain. However, the C-terminal kinase domain ("KD") construct (containing amino acids 1661-2386) did not exhibit dominant negative properties when overexpressed in wild-type cells, and failed to complement *rad3*⁻ mutants.

The above data suggests that overexpression of *rad3* alleles which contain the N-terminus but lack kinase activity will cause dominant negative phenotypes by titrating important interactors into nonfunctional complexes. It also implies that the N-terminus may be the site of an important protein interaction, possibly mediated by the leucine zipper motif.

- Deletion series into Nterm construct to localize regions important for dominant negative activity

In order to further localize the site of the interaction causing the dominant negative phenotype, I have performed a deletion series into the dominant negative "Nterm" construct. I have created constructs which overexpress *rad3* pieces 1-690 (N690), 1-541 (N541), 1-372 (N372), and 1-184 (N184). Interestingly, N690 retains the ability to cause dominant negative phenotypes in wild-type cells. However, N541 does not, despite the fact that it contains the leucine zipper motif. N372 and N184 cause novel phenotypes in wild-type cells: they cause the cells to become sick in the presence OR absence of DNA replication blocks. These constructs also both contain the leucine zipper motif, and their phenotypes make us wonder whether localization of these *rad3* polypeptides is important for their function. We have also specifically deleted the leucine zipper of the Nterm construct, which resulted in loss of the dominant negative function. This indicates that the leucine zipper does have a role in dominant negative function, although it is not sufficient for it. The Psite mentioned above also seems to be required for dominant negative function of the Nterminal construct: deletion or mutation of it also makes the Nterm construct unable to confer HU-sensitivity on wild-type cells.

- Examination of phenotypes of leucine zipper and Psite in full-length *rad3* constructs

I have also examined the phenotypes of leucine zipper deletion and the Psite deletions in full length constructs. Both of these constructs fail to complement *rad3Δ* cells, indicating again that these sites may have important roles in normal *rad3* function. Interestingly, the LZdel-*rad3* construct confers dominant negative properties on wild-type cells, while Pdel-*rad3* does not.

In the future, I am going to further my analysis of important regions necessary for Rad3p activity by making more deletions and mutations, and analyzing them for their ability to complement and confer dominant negative properties on wild-type cells. In collaboration with Sarah Evans, we will investigate the ability of the different alleles to demonstrate *in vitro* kinase activity.

Conclusions

I. Identification of gene products that interact with Rad3p

We have discovered genetic interactions between Rad3p and both *pol δ* and PCNA, as well as a physical interaction between Rad3p and PCNA. In future experiments, we hope to further understand exactly how these proteins might function together. For example, one model may be that *pol δ* or PCNA indirectly regulate Rad3p activity. When *pol δ* is mutated in the PCNA binding domain, Rad3p activity is unregulated (either relieved from inhibition or activated) and the checkpoint signal is enhanced. Others have hypothesized that there may be a link between PCNA and the checkpoint machinery (29). Some of my short-term experiments include examining whether the synthetic dosage phenotype, or elongation of *cdc6* alleles at the permissive temperature when *rad3+* is overexpressed, requires the checkpoint pathway to be intact.

II. Regulation of Rad3p protein and activity

In order to further our understanding of regulation of Rad3p protein and activity, we have initiated a systematic structure/function analysis of the *rad3* sequence. Our studies indicate that the leucine zipper motif, the kinase domain, and a site we call the "Psite," are all required for proper Rad3p function. We will be continuing our analysis of the mutants we have generated by measuring their phenotypes quantitatively by performing hydroxyurea time courses, radiation curves, and assessments of their effects on Rad3p kinase activity (using an assay developed by Sarah Evans in the lab).

We believe our studies are furthering our understanding of how Rad3p functions, and hope that our work will be used as a model for ATM.

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Appendix 1. Alignment of PCNA-binding motifs found in various proteins.

Sp Rad3	S S V K Q S L L L D G F F R W S
Hs p21	G R K R R Q T S M T D F Y H S K R R L
Mm p21	G R L R R Q T S L T D F Y H S K R R L
Hs Fen1	R Q G S T Q G R L D D F F K V T G S L
Sp Rad2	S K T I P Q G R L D S F F K P V P S S
Sc Rad27	L K S G I Q G R L D G F F Q V V P K T
Sp Rad13	Q F V G T Q S N L T Q F F E G G N T N
Sc Rad2	K K K G K Q K R I N E F F P R E Y I S
Hs XP-G	D A Q Q T Q L R I D S F F R L A Q Q E
Sp Cdc27	Q S K P Q Q K S I M S F F G K K
Sc Pol32	K R L K K Q G T L E S F F K R K A K